



## Short Communication

## Galleria mellonella larvae allow the discrimination of toxic and non-toxic chemicals

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## HIGHLIGHTS

- The acute toxicities of 19 chemicals were assessed using *G. mellonella* larvae.
- Globally Harmonised System category 5 chemicals were better predicted using *G. mellonella* larvae than cell culture systems.
- A more robust assessment chemical toxicity would take into account activity in cell cultures and in *G. mellonella* larvae.

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## ABSTRACT

The acute toxicities of 19 chemicals were assessed using *G. mellonella* larvae. The results obtained were compared against LD50 values derived from in vitro cytotoxicity tests and against in vivo acute oral LD50 values. In general, cell culture systems overestimated the toxicity of chemicals, especially low toxicity chemicals. In contrast, toxicity testing in *G. mellonella* larvae was found to be a reliable predictor for low toxicity chemicals. For the 9 chemicals tested which were assigned to Globally Harmonised System (GHS) category 5, the toxicity measured in *G. mellonella* larvae was consistent with their GHS categorisation but cytotoxicity measured in 3T3 or NHK cells predicted 4 out of 9 chemicals as having low toxicity. A more robust assessment of the likely toxicity of chemicals in mammals could be made by taking into account their toxicities in both cell cultures and in *G. mellonella* larvae.

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## 1. Introduction

Estimating the toxicity of chemicals, biocides, pesticides and cosmetics is essential to reduce the risk of the exposure of humans to hazardous materials that could cause illness (European Union, 2006). Apart from cosmetic testing, tests are typically carried out using mammals, providing an LD50 value. Increasingly, there are initiatives to reduce the use of mammals for testing. In vitro toxicity data towards cultured cells can be used to estimate the dose for subsequent rodent acute toxicity tests, and this methodology forms the basis of the acute toxicity Organisation for Economic Co-operation and Development (OECD) 129 method (Environment directorate joint meeting of the chemicals committee and the

working party on chemicals, 2010). This allows the use of mammals to be reduced, as a consequence of refined dose estimations. However, although cytotoxicity tests can reveal intrinsic cell sensitivity to the toxicity of chemicals, factors associated with metabolism of the compound such as rates of absorption, biotransformation, distribution and excretion may not be simulated in cell culture systems (Schrage et al., 2011; van Vliet, 2011). Consequently, the toxicity of some chemicals in mammals is not well predicted from cell culture data (Environment directorate joint meeting of the chemicals committee and the working party on chemicals, 2010; Schrage et al., 2011; van Vliet, 2011). Furthermore, differences occur between cytotoxicity test results in different laboratories. This may reflect differences in the cell line used, preservation and culture methods and passage number. These limitations are highlighted in the OECD 129 guidance document (Environment directorate joint meeting of the chemicals committee and the working party on chemicals, 2010) which

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states “The limitations of the in vitro methods are largely due to the differences between whole animal and cell culture systems”

Consequently there is a requirement for tests that can better predict toxicity in mammals. *Galleria mellonella* (wax moth) larvae are considered to be non-animal technologies. However, they reflect aspects of the complexity present in mammals and are accepted as an ethical alternative for research (Lionakis, 2011; Champion et al., 2016; Tsai et al., 2016). For example, *G. mellonella* possess an innate immune system that is structurally and functionally similar to that of mammals (Browne et al., 2013; Wojda, 2017). In the UK and other countries studies using *G. mellonella* larvae are not regulated, reducing the costs associated with studies and enabling the system to be used flexibly. In addition, the ability to use large groups of larvae can increase the statistical power of studies. The larvae used are 6th instar stage and do not feed. Therefore maintenance is simple. *G. mellonella* are being used widely to investigate mechanisms of bacterial and fungal pathogenicity, and the efficacy and pharmacokinetics of antimicrobials (Lionakis, 2011; Champion et al., 2016; Tsai et al., 2016).

More recently there are preliminary reports that *G. mellonella* can be used to test the toxicity of chemicals (Megaw et al., 2015; Dolan et al., 2016; Maguire et al., 2016). In one study, *G. mellonella* larvae were used to measure the relative toxicity of 8 food preservatives and a correlation between the LD50 values in *G. mellonella* larvae and in rats was demonstrated (Maguire et al., 2016). Here we set out to test the potential to use *G. mellonella* larvae to predict the acute toxicity of a panel of compounds in mammals.

## 2. Material and methods

### 2.1. Chemicals

Most chemicals were obtained from Sigma-Aldrich (Poole, UK). Acetonitrile, glycerol, sodium dichromate dehydrate, sodium hypochlorite and xylene were obtained from Fisher Scientific (Loughborough, UK). Chloramphenicol was obtained from Acros Organics (Loughborough, UK).

### 2.2. Injection of *Galleria mellonella* larvae

*G. mellonella* larvae (TruLarvTM) were purchased from Biosystems Technology (Exeter, UK) (Champion et al., 2009; Wand et al., 2011). Larvae (0.2 g) were injected with 10 µL of chemical into the front proleg using a micro-syringe (Hamilton Ltd). For each chemical 6 dilutions were made and each dilution injected into a groups of 10 larvae. Larvae were incubated at room temperature (20–25 °C) and mortality scored at 24 h, 48 h and 72 h. Death was defined as complete loss of mobility including following a physical stimulus using a plastic pipette.

### 2.3. Calculation of LD50

In order to analyse mortality, a probit Model was used:  $y = \Phi(a + b \cdot \text{Log}_{10}(\text{conc}))$ , where conc is concentration,  $\Phi$  is the standard normal distribution and  $y$  is the proportion of events which occurred. Parameters  $a$  and  $b$  were estimated as well as  $\text{Log}_{10}(\text{LD}_{50}) = -a/b$ . To evaluate the dose response relationship, the 95% confidence interval for  $b$  (slope) was determined. The calculation of LD50 provides a value in mg/mL, the LD50 in mg kg<sup>-1</sup> was determined by multiplying by 50, assuming a typical weight of 0.2 g per larvae.

### 2.4. Cytotoxicity and oral toxicity in mammals

The toxicities of chemicals in human epidermal keratinocyte cells (NHK cells), in murine fibroblast cells (3T3 cells) or oral toxicity in rats was taken from the OECD Guidance on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests (Environment directorate joint meeting of the chemicals committee and the working party on chemicals, 2010). The LD50 doses of chemicals in mammals, extrapolated from IC50 values in cell culture, were calculated as previously described (Environment directorate joint meeting of the chemicals committee and the working party on chemicals, 2010).

## 3. Results and discussion

### 3.1. Effects of solvents on *G. mellonella* larvae

We first tested the responses of *G. mellonella* larvae to ethanol, water or DMSO solvents. No deaths were recorded in larvae dosed with SDW. All larvae were killed after the injection of 80% DMSO but not 50% DMSO. 30% (v/v) ethanol was tolerated, but 2 of 10 larvae dosed with 40% ethanol died. For subsequent studies we ensured that the concentrations of solvents given to larvae did not exceed 50% (v/v) DMSO or 30% (v/v) ethanol.

### 3.2. Testing chemical toxicity in *G. mellonella* larvae

We selected 30 chemicals for toxicity testing in *G. mellonella* larvae. Three chemicals selected for testing (5-aminosalicylic acid, propylparaben and xylene) could not be solubilised and could therefore not be injected. The toxicity of 8 chemicals could only be estimated due to limited solubility (Table 1A). A dilution series of the remaining 19 chemicals was made to provide concentrations bracketing the expected LD50 dose. Each dilution was injected into a group of *G. mellonella* larvae, mortality recorded and the LD50

**Table 1**  
Calculated LD50 values, for A) compounds with limited solubility B) soluble compounds.

	Chemical	LD50 (mg kg <sup>-1</sup> )	Solvent
A	phenylthiourea	>300	30% DMSO
	aminopterin	>375	30% DMSO
	caffeine	>1000	SDW
	sodium oxalate	>1000	SDW
	Chloramphenicol	>1500	30% ethanol
	Cycloheximide	>1600	SDW
	valporic acid	>3000	30% ethanol
	hexachlorophene	NR	10% ethanol
	disulfoton	1.55	SDW
	cadmium chloride	4.65	SDW
B	triphenyltin hydroxide	31.6	50% DMSO
	Phenol	97.6	SDW
	Digoxin	469	30% DMSO
	Atropine sulfate	582	SDW
	Colchicine	1492	SDW
	Lindane	1781	10% DMSO
	Acetaminophen	2124	30% DMSO
	citric acid	2458	SDW
	trichloroacetic acid	2777	SDW
	sodium hypochlorite	3245	SDW
	sodium dichromate dihydrate	5378	SDW
	procinamide HCl	6140	SDW
	2-propanol	12079	SDW
	dibutyl phthalate	17,200	SDW
	Dimethylformamide	28,214	SDW
	Glycerol	29,495	SDW
	Acetonitrile	37,520	SDW

NR; did not give a typical dose concentration response.

values calculated (Table 1B).

### 3.3. Prediction of toxicity from studies in *G. mellonella* larvae and in cell cultures

We next considered toxicities according to the GHS categorisation of chemicals. This system places chemicals into one of five categories (Fig. 1a and Table S1) ranging from low toxicity (GHS category 5, LD50 2000–5000 mg kg<sup>-1</sup>) to highly toxic (GHS category 1, LD50 < 5 mg kg<sup>-1</sup>). For the 9 chemicals tested which had been assigned to GHS category 5, the toxicity measured in *G. mellonella* larvae was consistent with the GHS categorisation (Fig. 1b). In comparison, cytotoxicity measured using 3T3 or NHK cells predicted 4 out of 9 chemicals in GHS category 5 as having low toxicity with the remaining 5 chemicals being predicted as weakly toxic or very toxic. A previous study (Schrage et al., 2011) where 79 GHS category 5 chemicals were tested also highlighted the poor correlation of oral toxicity in rats with in vitro cytotoxicity. In this previous study, of the 79 chemicals tested only 6 (8%) were found to have low toxicity towards cell cultures, the remainder being more toxic. This might reflect the inability of cell cultures to detoxify or excrete cytotoxic chemicals. These findings are important, because the incorrect assessment of these chemicals could trigger studies using mammals to evaluate oral toxicity and could result in the unnecessary testing of compounds in mammals.

For chemicals tested in this study where acute oral LD50 values were less than 2000 mg kg<sup>-1</sup> (GHS categories 1–4), testing in *G. mellonella* larvae showed 8 out of 10 chemicals to be correctly predicted. In comparison, cytotoxicity methods predicted 10 out of 10 chemicals as being classified in GHS categories 1–4.

Overall, testing in *G. mellonella* larvae classified 11 out of 19 chemicals (58%) in the same GHS class as measured in rats. Cytotoxicity towards NHK cells classified 11 out of 19 chemicals (58%) in the same hazard class as measured from acute oral LD50 tests in rats. Cytotoxicity towards 3T3 cells classified 9 out of 19 chemicals (47%) in the same hazard class.

Some chemicals are converted into other toxins as a

consequence of mammalian metabolism, and especially via the cytochrome P450 enzymes. There is some data indicating that the cytochrome P450 enzymes from insects behave like their counterparts in mammals (Feyereisen, 1999). For example, converting caffeine into theobromine, paraxanthine and theophylline (Coelho et al., 2015). However, there are also examples of differences. For example, in mammals acetonitrile is metabolised to cyanide by cytochrome P450 enzymes (Pozzani et al., 1959), but this chemical was essentially non-toxic in *G. mellonella*. Since insects are susceptible to killing by cyanide (Bond, 1962) this suggests that they do not metabolise this chemical in the same way as mammals.

Toxicity measurements in cell cultures or in *G. mellonella* larvae both have limitations. However, there are differences in the ease with which the tests can be carried out. Significant preparation time is required for the propagation of cells in the laboratory before use, whereas *G. mellonella* larvae can be purchased and used on the same day.

## 4. Conclusions

This study investigated the suitability of *G. mellonella* larvae to measure the acute toxicity of chemicals compared to cell culture systems. In general, cell culture systems overestimated the toxicity of chemicals, especially low toxicity chemicals. In contrast, toxicity testing in *G. mellonella* larvae was found to be a reliable predictor for low toxicity chemicals. We believe that a more robust assessment of the likely toxicity of chemicals in mammals could be made by measuring toxicity in both cell culture and in *G. mellonella* larvae. Further studies in *G. mellonella* larvae to establish repeatability and reproducibility of tests and to investigate a wider panel of chemicals are warranted.

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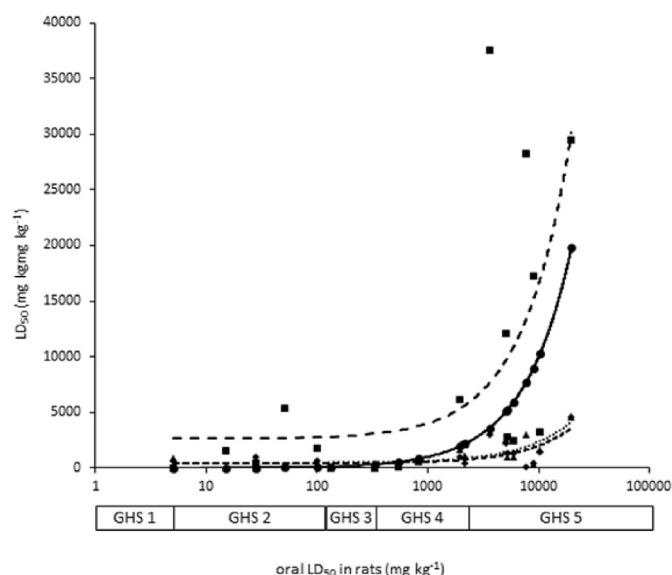
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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.chemosphere.2018.01.175>.

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**Fig. 1.** Linear trendlines showing the relationship of oral toxicity in rats (x axis) with oral toxicity measured in rats (●), in *G. mellonella* larvae (■) or extrapolated from toxicity data in 3T3 (◆) or NHK (▲) cell cultures. The panels below the X-axis show the range of toxicity of GHS category 1 (<5 mg kg<sup>-1</sup>); GHS category 2 (5–50 mg kg<sup>-1</sup>); category 3 (50–300 mg kg<sup>-1</sup>); category 4 (300–2000 mg kg<sup>-1</sup>); category 5 (>2000 mg kg<sup>-1</sup>).

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